BIOSPECIFIC ASSAY METHOD

This invention relates to a biospecific assay method in
which the analyte to be assayed is bound to the surface of
microparticles through a bioaffinity reactant binding the
aforesaid analyte, and the amount of analyte bound to
5   individual microparticles is determined.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to
illuminate the background of the invention, and in
particular, cases to provide additional details respecting
10   the practice, are incorporated by reference.

Traditionally, radioimmunoassays were in the beginning
performed in solution using test-tubes and complicated
separation methods. With the advent of solid phase
technology radioactive isotopes have been replaced by
15   diverse labels, and especially monoclonal antibodies have
been used in an increasing number of applications.

The aforementioned improvements have made a wider range of
traditional immunoassays and other bioaffinity assay
methods available (e.g. Alternative Immunoassays, W.P.
20   Collins, Wiley, Chichester 1985, and Luminescence
Immunoassay and Molecular Applications, Ed. Knox van Dyke,
CRC Press, Boston 1990, and R.P. Ekins et al., Clin. Chem.
1991; 37:1955 - 1967). Solid phase assays have become
routine assay methods both in the field of competitive and
25   non-competitive assays, and the traditional test-tube has
often been replaced by alternative solid phase facility.
The microtiter plate was introduced as a solid phase for
routine hormone assays in 1984 when Wallac Oy (Turku,
Finland) introduced their DELFIA technology. Other
30   commercial enterprises have also adopted the microtiter
plate in similar routine assays, which, however, are based

on different labelling technologies.

Microparticles manufactured of different materials have for
some time been available as an alternative solid phase for
bioaffinity assays (e.g. Molday W.J. et al., J. Cell.
Biol.: 1975: 64, 75, and Nustad K. et al.: Microspheres.
Medical and biological applications, Ed. A. Rembaum and
Z.A. Tökes, Boca Raton, Florida, CRC Press 1988). Compared
to microtiter plates the microparticles offer several
advantages, for instance an immobilization method for
bioaffinity reactants more easily adaptable to production
scale, and a greater reaction velocity obtainable when
using microparticles. The capacity of the microsphere-
associated solid phase is also easily controllable by
simply dispensing an amount of particles optimal for the
method in question. The greatest disadvantages of
microparticles have been traditionally associated with
liquid handling (e.g. washes). With the introduction of
magnetic microspheres this problem has been partly
overcome, and in recent years an increasing number of
commercial automated immunoassay methods have been based on
their use. Nowadays novel manufacturing methods have
allowed the production of microparticles of exact and
reproducible size (e.g. of a diameter of 0.04 - 100 µm).
Also the immobilization of the bioaffinity reactants onto
the microparticles can be effected by various chemical
methods.

The competitive biospecific assay method may be described
as follows: The microparticles are coated with the
bioaffinity reagent A, for whose binding sites, on the one
hand, the analyte contained in the sample competes, and on
the other hand, the bioaffinity reagent B, in this case an
appropriately labelled analyte, also competes for the said
binding sites. In the case of immunoassay, bioaffinity
reagent B will be a labelled antigen, the antigen being the
same as the antigen contained in the sample or to a
suitable labelled derivative thereof. Because the analyte

contained in the sample and the labelled analyte compete for the binding sites of the bioaffinity reactant A, the amount of label bound to the microparticle via reactant A, and consequently the signal from the microparticle, will be
5 inversely related to the concentration of the analyte in the sample.

The non-competitive biospecific assay method may be described as follows: The microparticles are coated with the bioaffinity reactant A, whose binding sites only bind
10 the analyte contained in the sample. The bioaffinity reactant B, which in this case is a suitably labelled reactant directed against the analyte contained in the sample, is bound by the analyte, which is linked to the microparticle via the bioaffinity reactant A. In the case
15 of immunoassay, bioaffinity reactant B will be a labelled antibody directed against the antigen contained in the sample. The amount of label bound to the microparticle via reactant A, and consequently the signal strength from the microparticle, will be directly related to the
20 concentration of the analyte in the sample.

The sensitivity of the competitive and non-competitive immunoassay has been extensively discussed earlier (Ekins R.P. et al., Pure and Appl. Chem., 1985; 57: 473 - 482, and Ekins R.P. et al., Clin. Chem., 1991; 37: 1955 - 1967). As
25 regards the present invention, it is essential to consider the factors affecting the sensitivity and functionality of each assay principle.

Maximal sensitivity in a competitive immunoassay when using an antibody (a bioaffinity reactant) with an affinity
30 constant K will be obtained by dividing by K the relative error of the signal strength at zero concentration. The error of the signal strength will be affected by two components, an experimental error component due to pipetting and other manipulations, and a signal strength
35 measurement component, e.g. the statistical error of signal

strength measurement. For the sake of simplicity, let us assume that the error due to signal strength measurement is 0, which is usually the case when the specific activity of the label is high. The maximal attainable sensitivity of

5   the competitive assay is now ϵ/K where ϵ is the combined relative error due to experimental factors. Let us assume, for instance, that the experimental error is of the order of 1%; the maximal attainable sensitivity then, using an antibody with an affinity constant as high as possible, for

10   instance,
10^{12} l/mol, is of the order of 0.01 picomol/l. The sensitivity definition also demonstrates that unless the experimental error can be almost completely eliminated (< 0.1%), there is no sensitivity advantage to be gained in

15   using a label with extremely high specific activity.

Likewise, the factors affecting the sensitivity of the non-competitive are the following:
a) the relative error of the signal strength (α) when the analyte is omitted, i.e., the error due to non-specific

20   binding of the labelled antibody (bioaffinity reactant)
b) the relative amount of non-specifically bound labelled antibody (k), and
c) the affinity constant (K) of the antibody
The sensitivity of the non-competitive assay is then

25   $k/K \times \alpha$. The sensitivity of both competitive and non-competitive assay is inversely related to the affinity constant of the antibody used, but the non-competitive assay is affected by the relative error due to, on the one hand, non-specific binding of the labelled antibody and on

30   the other hand, the relative binding of the labelled antibody, both of which could be assigned, for instance, a value of 1%. The maximal sensitivity of the non-competitive assay is then $0.0001 \times 1/K$ or 0.1 fmol/l, if the affinity constant of the antibody used is 10^{12} l/mol, which is

35   usually considered to be the highest possible value. In a non-competitive assay it is advisable to consider the specific activity of the label, because it affects factors

a) and b), and offers a possibility to improve the
sensitivity of the assay.

In assays based on bioaffinity reactions factors
structurally limiting the sensitivity of various
5 determinations must also be considered; this is also
essential as regards the present invention, because
measurements will be taken from individual microparticles
with extremely small amounts of analytes present.

SUMMARY OF THE INVENTION

10 This invention relates to a biospecific assay method, in
which method microparticles coated with the bioaffinity
reactant A binding the analyte to be assayed; the sample to
be analysed, and the labelled bioaffinity reactant B are
mixed. After the binding reaction the signal strength from
15 the labelled bioaffinity reactant B bound to the
microparticles is quantitated for the determination of the
analyte concentration of the sample. According to the
present invention such an amount of sample and
microparticles is used in the assay that after binding of
20 the analyte of the sample to the said amount of
microparticles, each individual microparticle will emit
such a signal strength as to allow the measurement of the
analyte concentration in the sample over the whole range of
typical analyte concentrations, and the signal strength
25 from each microparticle will be measured separately.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a calibration curve for hCG construed by
measuring individual microparticles by time-resolved
microfluorometry

30 Figure 2 shows the signal strength versus analyte
concentrations for different amounts of microparticles
(measured from individual microparticles)

Figure 3 shows the signal strength versus amount of
microparticles per assay for two different amounts of
analyte DNA present in the reaction (measured from
individual microparticles). The curve indicated by closed
5   squares represents 10^9 molecules per reaction and the curve
indicated by open squares represents 10^{10} molecules per
reaction.

DETAILED DESCRIPTION OF THE INVENTION

The present invention allows the assay of the analyte in
10   the sample at a minimal analyte concentration and in a
minimal volume by selecting such an amount of
microparticles that allows the measurement of the analyte
bound to the surface of an individual microparticle, by the
sensitive label technology used. The biospecific reaction
15   may be an immunoassay, a nucleic acid hybridization assay,
a ligand-lectin assay or a ligand-receptor assay. The
bioaffinity reaction may be advantageously performed on a
microparticle having a diameter of less than 1 mm. The
analyte concentration on the surface of the microparticle
20   is determined by means of the labelled bioaffinity reactant
B, the label being one emitting fluorescence, time-resolved
fluorescence, chemiluminescence or bioluminescence. The
present invention allows the measurement of both the
highest and the lowest concentrations of analyte from
25   individual microparticles. The sensitivity of the assay and
the measurement range are controlled by the amount of
microparticles used in the assay.

The present invention may be applied to many types of
biospecific affinity reactions, although the most commonly
30   used is the immunochemical reaction. This is why the
present invention is predominantly described as a
immunochemical assay. The relevant biospecific affinity
reactions (immunoreactions) are either competitive or non-
competitive. Both immunoassay principles are commonly

known. The factors affecting the sensitivity of the said
immunoassay methods are known and the optimal sensitivity
of the assays can be calculated. The highest attainable
sensitivity in the competitive assay is about 10 fmol/l.

5 The sensitivity cannot be improved by increasing the
specific activity of the label used. Sensitivity can be
improved to a certain degree by using a minimal antibody
concentration. In the competitive assay the signal strength
from the label to be measured is inversely related to the

10 concentration of the sample (analyte) to be assayed.

The highest possible sensitivity attainable in the non-
competitive assay is about 0.1 - 0.01 fmol/l. The
sensitivity may be enhanced if a label with a very high
specific activity is available. The sensitivity can be

15 improved up to a certain limit by using the highest
possible concentration of labelled antibody if the
background of the assay (non-specific binding to the
surface) remains low. In the non-competitive assay the
signal strength from the label to be measured is directly

20 related to the concentration of the sample (analyte) to be
assayed.

In the competitive immunoassay the assumption may be made
that the measuring range of the analyte to be assayed is
typically 100-fold, i.e., so wide that the ratio of the

25 highest to the lowest measured value is 100. In the
competitive assay the lowest concentration gives the
strongest signal and the highest concentration gives the
weakest signal. In the competitive assay based on the use
of microparticles, in which the measurement is taken from

30 individual particles, one must be able to measure the
signal strengths from both the lowest and the highest
concentration. If we assume a measuring range of 1 - 0.01
pmol/l for the sample to be assayed, then a concentration
of 0.01 pmol/l corresponds to the strongest signal, and 1

35 pmol/l to the the weakest signal, respectively. The
concentration of the analyte of the example is 1 - 0.01

attomol/µl which means that when using a sample volume of 1
µl (1 - 0.01 attomol of analyte to be assayed) one must be
able to measure, from a single microparticle, both the
highest (0.01 attomol of sample) and the lowest (1 attomol
5   of sample) signal strength. This means that when the
concentration of the competing analyte is low, the highest
possible number of analyte, i.e. bioaffinity reactant
molecules will specifically bind to the surface of a single
microparticle coated with a specific antibody, i.e. a
10 second bioaffinity reactant. The lowest analyte
concentration means that a sample volume of 1 µl contains
approximately 6,000 molecules of the analyte to be assayed,
while the highest concentration corresponds to
approximately 600,000 molecules in a microlitre,
15 respectively. It is typical of the competitive assay that
the signal strength decreases from about 100% to about 5 -
25% with increasing analyte concentration. The amount of
the microparticles and of the analyte-specific antibody
coated onto them will be adjusted to a minimal sample
20 volume in order to allow a replacement (of labelled analyte
by the analyte to be assayed) of 75 - 95% at the highest
sample concentration. This still allows the remaining
signal strength to be reproducibly measured, by the label
technology used, from the surface of individual
25 microparticles, and at the same time, the signal strength
(due to the specific binding of labelled analyte to the
coated microparticle) corresponding to the lowest analyte
concentration will not exceed the binding capacity·of
individual particles. The measuring range is adjusted for
30 different assays by altering the amounts of the
microparticles and antibodies used in the assay method in
such a manner that measurements can always be taken from
individual microparticles.

The sensitivity of the non-competitive immunoassay is, at
35 best, 0.01 - 0.1 fmol/l, and the measuring range is
typically more than 100-fold, i.e. the ratio of the highest
to the lowest measurement value is greater than 100. The

signal strength measured in the assay is directly related to the concentration of the analyte to be assayed. For a required sensitivity of 0.01 pmol/l for the analyte to be assayed, and assuming a measuring range of 1,000-fold, a

5 sample volume of 1 µl will contain 0.01 - 10 attomol of the analyte to be assayed. If the sensitivity of the label technology is about 6,000 molecules per one microparticle, and with a signal strength directly related to the concentration of the analyte, it is possible for one

10 microparticle to bind the analyte contained in 1 µl, and the amount of the analyte can be measured. The amount of microparticles used in the assay can be increased without changing the sample volume, if the sensitivity of the label techonology is better or if a greater number of labelled

15 molecules are linked to the labelled analyte-specific antibody (bioaffinity reactant). Accordingly, if the sensitivity of the label technology is lower, the sensitivity of the assay will decrease or the amount of analyte required on the surface of a single microparticle

20 must come from a larger sample volume, which however limits the measurement range. The amount of the microparticles used in the assay, coated with the analyte-specific antibody or bioaffinity reactant as well as the amount of the analyte per microparticle will be adjusted so that a

25 minimal concentration and volume of the analyte will contain enough analyte for binding to the surface of individual microparticles, and enough for measurement from individual microparticles by means of a labelled specific antibody (labelled bioaffinity reactant) and with the

30 sensitive label technology used. The measurement range required and the sensitivity of the measurement will be controlled by adjusting the amount of microparticles used in the assay and by adjusting sample volume, if needed.

Other specific bioaffinity assays such as nucleic acid
35 hybridization assays, ligand-lectin assays and ligand-receptor assays are comparable to the immunoassay methods described above.

Individual microparticles can be assayed with e.g. a flow
cytometer, time-resolved microscope or time-resolved
microfluorometer or with other measuring instruments based
on the use of time-resolved technology (US 5,028,545;
5   Seveus L et al., Cytometry 13: 329 - 338 (1992).

Microparticles may also eventually be used in
multiparameter assays, in which several analytes are
simultaneously assayed in the same sample volume. In the
multiparameter assay a mixture of microparticles is used,
10   in which the particles assaying for different analytes are
coated with analyte-specific bioaffinity reactants. When
the measurement is taken the analyte-specific microparticle
categories are identified on the basis of e.g. size,
fluorescence, time-resolved fluorescence, chemiluminescence
15   or bioluminescence. The multiparameter assays using
microparticles, based on a bioaffinity reaction are
performed according to the present method. In the case of
several analytes being assayed simultaneously the amount of
the analyte-specific microparticles used in the assay is
20   adjusted according to analyte following the above
principles.

The present invention will also be illustrated by the
following non-restrictive examples.

Example 1

25   Quantitation of label (fluorescent Eu chelate) per
microparticle and immunoassay measurement from individual
microparticles using as a model the non-competitive hCG
assay and measurement based on time-resolved fluorescence.

The hCG (hCG = human chorionic gonadotrophin) assay has
30   been performed as a non-competitive immunoassay with a
specific monoclonal antibody against the ß subunit being
biotinylated and bound to 20 µm microparticles coated with

streptavidin. The labelled bioaffinity reactant has been a
monoclonal antibody labelled with a fluorescent Eu chelate,
specifically recognizing the α subunit of hCG. The
immunoassay has been performed in one step, during which

5 the antibody-coated microparticles, the hCG calibration
standard (2 - 10,000 U/l, where U/l = unit/l) and the
labelled antibody are all present simultaneously. After the
immunoassay the number of microparticles has been checked
by counting, and the Eu concentration has been determined

10 from the known microparticle amount by using the DELFIA
assay solution, which then allows the calculation of the
amount of Eu molecules per microparticle. In addition
individual microparticles have been measured by time-
resolved microfluorometry which allows the measurement of

15 the concentration per microparticle of the hCG-specific
antibody labelled with fluorescent Eu chelate. The hCG
assay presented as an example has not been completely
optimized.

Results: Table 1 shows the results of the hCG assay when
20 using 20 μm microparticles. In these assay conditions the
lowest hCG concentration resulted in about 5,000 Eu
molecules per microparticle, the variation within the
experiment still being wide. In addition the measuring
range is at least 1,000-fold. Figure 1 shows a calibration
25 curve for hCG construed by measuring individual
microparticles by time-resolved microfluorometry.

Example 2

The effect of the amount of microparticles coated with
antibody (bioaffinity reactant) on the sensitivity of the
30 assay using as a model the non-competitive hCG immunoassay
and the measurement of individual microparticles with a
microfluorometer, based on time-resolved fluorescence.

The immunoassay of hCG was performed according to the
preceding example. A total volume of 20 μl, three

concentrations of calibration standard (0, 250, and 2,500 U/l) and three different amounts of microparticles were used in the assay. After the immunoreaction individual microparticles were measured by time-resolved

5   microfluorometry.

Results: Table 2 and Figure 2 show the effect of the amount of microparticles on the signal strength (photons/s/particle) measured from individual microparticles; with a smaller amount of microparticles the

10 hCG (analyte) in the sample will be bound in a higher concentration onto the surface of individual microparticles; the sensitivity of the assay and the measurement range may be controlled, if needed, by the amount of the microparticles and the sample volume, so as

15 to allow measurement from individual microparticles. The reproducibility of the assay can be improved by measuring a greater number of individual microparticles.

Example 3

The effect of the amount of microparticles on nucleic acid

20 hybridization reaction using two different concentrations of target nucleic acid.

The microparticles, of a diameter of 20 µm, were coated with streptavidin and used in a hybridization assay where the target was biotin-labelled synthetic oligonucleotide

25 and the probe a synthetic oligonucleotide labelled with a fluorescent Eu chelate. The experiment was performed using two different amounts of target oligonucleotide (10^9 and 10^{10} molecules). The total assay volume was 20 µl and 2.0 ng of probe labelled with the fluorescent Eu chelate was used

30 in each assay. The amount of microparticles used per experiment varied from 10 - 10,000 for each target oligonucleotide concentration. After the hybridization reaction the Eu concentration was measured from the surface of the microparticles by time-resolved microfluorometry.

Results: Table 3 and Figure 3 show the effect of the amount
of microparticles on the signal strength measured from
individual microparticles; with a smaller amount of
microparticles the amount of the target oligonucleotide in
5   the sample will bind in higher concentration onto the
surface of individual microparticles; again the sensitivity
of the assay and the measurement range may be controlled,
if needed, by the amount of microparticles used and by the
sample volume so as to allow measurement from individual
10 microparticles.

A specialist in the field will appreciate that the
different applications of the said invention may vary
within the scope of the claims. It will be appreciated that
the methods of the present invention can be incorporated in
15 the form of a variety of embodiments, only a few of which
are disclosed herein. It will be apparent to the artisan
that other embodiments exist and do not depart from the
spirit of the invention. Thus, the described embodiments
are illustrative and should not be construed as
20 restrictive.

TABLE 1

Calculated Eu amounts in hCG-standards using 20 μm particles. Great assay amount.

hCG U/l	signal strength photons/s	particles	Eu fmol	Eu fmol/cm²	Eu mol/part.	Eu molecules/ particle	average	% CV
2	118	9624	0.082	0.678	8.52×10^{-21}	5130	5130	
	<0	10451						
	<0	7444						
10	130	7970	0.09	0.899	1.13×10^{-20}	6805	13881	72.09
	<0	6917						
	306	6090	0.212	2.77	3.48×10^{-20}	20957		
100	817	7293	0.566	6.176	7.76×10^{-20}	46732	58696	20.21
	869	6165	0.603	7.783	9.78×10^{-20}	58897		
	1030	8195	0.714	6.933	1.17×10^{-19}	70459		
1000	12955	7293	9.98	97.99	1.23×10^{-18}	740727	618677	17.21
	13142	9624	9.11	75.33	9.47×10^{-19}	570299		
	11474	8797	7.96	72.01	9.05×10^{-19}	545006		
5000	78247	5940	54.26	726.92	9.14×10^{-18}	5504263	4428302	21.13
	71234	7820	49.4	502.7	6.32×10^{-18}	3806011		
	70166	7368	48.66	525.55	6.60×10^{-18}	3974632		
10000	146072	6090	101.29	1323.55	1.66×10^{-17}	9996802	6989043	45.32
	112916	7368	78.3	845.67	1.06×10^{-17}	3683500		
	113853	6541	78.95	960.5	1.21×10^{-17}	7286826		

CV = coefficient of variation

TABLE 2

Effect of the amount of microparticles on the signal strength

hCG U/l	particles	signal strength photons/s/part.	CV %
0	100	252	6.5
250	100	490	26.15
2500	100	1671	23.1
0	1000	277	10.39
250	1000	345	15.76
2500	1000	418	19.98
0	10000	278	7.05
250	10000	366	23.34
2500	10000	401	38.5

Total assay volume 20 µl, of which standard = 5 µl. Size of microparticles 20 µm. Microfluorometry, laser excitation, attenuation 1:100.

TABLE 3

amount of particles	amount of analyte molecules	signal strength photons/s/part.	CV %
10000	10^3	497	34
1000	"	1005	48
100	"	1885	22
10	"	3852	24
no analyte/100		1358	40
10000	10^{10}	1622	31
1000	"	5613	25
100	"	13236	33
10	"	15594	45
no analyte/100		1887	43